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Abstract

The purpose of this work was to investigate the functional interactions between laminin (LN)-10, integrin $\alpha\beta 3$ and matrix metalloproteinase (MMP)-9 in the metastasis of breast tumors to bone. Whilst both $\alpha\beta 3$ and MMP-9 have been implicated in bone metastasis, their relationship to LN-10 in this process remains largely unknown. We hypothesized that LN-10 contributes to breast cancer metastasis through direct interaction with $\alpha\beta 3$ and modulation of MMP-9 expression. Using a novel immunohistochemistry protocol, we found that aggressive 4T1.2 primary tumors and lung and bone metastases express high levels of LN-10 ($\alpha 5$ chain) but not LN-1 or LN-5. LN-10 is a potent adhesive substrate for mammary carcinoma cell lines irrespective of their metastatic potential but selectively promotes $\alpha\beta 3$ -dependent migration and MMP-9 expression of bone metastatic lines. Migration on LN-10 is inhibited by blocking antibodies directed against $\beta 3$ integrin, specific $\alpha\beta 3$ -binding LN-10 peptides or by down-regulation of MMP-9 expression in bone metastatic cells. The anti-metastatic potential of LN peptides is currently being tested in vivo. These findings suggest that LN-10 may have prognostic and/or therapeutic value for the treatment of metastatic breast tumors.

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INTRODUCTION

Breast cancer kills more women than any other cancers in developed countries. Whilst breast cancer is curable when detected early, current treatments are mainly palliative once the disease has spread to distant organs (1). We proposed to investigate the interactions between laminin (LN)-10, integrin $\alpha v \beta 3$ and matrix metalloproteinase (MMP)-9 and their role in breast cancer metastasis to bone, the most common site of metastasis for breast tumors. This research project was motivated by earlier work in our laboratory investigating tumor-stroma interactions and factors implicated in metastasis to bone. Using a unique syngeneic mouse model of spontaneous breast cancer metastasis developed in our laboratory (2,3), we identified a number of genes differentially expressed between highly/bone metastatic tumor lines and weakly metastatic lines. Of particular relevance to this research project, these studies identified integrin $\alpha v \beta 3$, MMP-9 and two subunits of LN-10 ($\alpha 5$ and $\beta 1$ chains) as being more highly expressed in primary tumors derived from bone metastatic lines compared to those derived from weakly and non-metastatic lines.

Whilst $\alpha v \beta 3$ and MMP-9 have been previously implicated in the metastasis of breast tumors to bone, their mechanism of action is incompletely understood. Stromal $\alpha v \beta 3$ integrin involvement in breast cancer progression has received significant attention due to its well-recognized role in osteoclast-mediated bone resorption (4) that invariably accompanies breast cancer metastasis to bone (5). The role of tumor-associated $\alpha v \beta 3$ is less clear but is believed to contribute to bone metastasis in part by mediating tumor cell arrest in blood flow through interaction with platelets (6) as well as promoting adhesion to and migration on bone matrix proteins through interactions with MMP-9 (7). Most investigations on the role of tumor $\alpha v \beta 3$ in breast cancer metastasis have focussed on classical substrates for this integrin including fibronectin, vitronectin and osteopontin but its relationship to LN-10, an abundant extracellular matrix (ECM) protein present in epithelial and vascular basement membranes (BM) of normal breast, remains largely unexplored. The recent demonstration that LN-10 interacts with $\alpha v \beta 3$ in ECV304 carcinoma cells to promote their migration and growth factor-induced proliferation established LN-10 as a novel functional substrate for this integrin (8). Surprisingly, whilst the functional role of LN-10 in tumor progression is well supported in other tissues (9-11), its specific role in breast cancer metastasis to bone has not been investigated. LN-10 is a proliferative and migratory substrate for a variety of epithelial tumor cells *in vitro* and specific peptide sequences present in LN-10 were recently reported to be potent inducers of MMP-9 (12) or to possess anti-angiogenic properties and to inhibit melanoma metastasis *in vivo* (11). Based on these observations, we hypothesized that LN-10 may contribute to breast cancer metastasis to bone through direct interaction with integrin $\alpha v \beta 3$ and modulation of MMP-9 expression/activity.

Thus the overall objectives of this project were to determine whether LN-10 is a functional adhesive substrate for mammary carcinoma cells and selectively promotes migration of bone metastatic variants expressing $\alpha v \beta 3$ integrin and MMP-9. In addition, we attempted to provide evidence that interfering with LN-10- $\alpha v \beta 3$ interactions has the potential to inhibit breast cancer metastasis to bone. To achieve these aims, we set out to perform the following tasks:

- 1- Confirm the differential expression of LN-10 protein ($\alpha 5$ chain) in mammary carcinomas of varying metastatic potential and investigate their adhesive and migratory responses to LN-10 and the role of $\alpha v \beta 3$ integrin on these responses
- 2- Determine the involvement of MMP-9 in LN-10-mediated migration
- 3- Provide evidence for specific interactions between tumor $\alpha v \beta 3$ integrin and endothelial LN-10 during intra/extravasation process
- 4- Screen LN-10 peptides for their migration inhibitory activity *in vitro* and test the effect of selected LN-10 peptides for their ability to suppress breast cancer metastasis to bone *in vivo*.

BODY

Task 1: (a) Confirm the differential expression of LN-10 ($\alpha 5$ chain) in mammary carcinomas of varying metastatic potential and (b) investigate their adhesive and migratory responses to LN-10 and the role of $\alpha v \beta 3$ integrin on these responses

a) expression of LN α chains in primary breast tumors and metastases

LNs are heterotrimers of α , β and γ chains that combine to form at least 15 isoforms (13-15). At least three isoforms of LN are present in the subepithelial basement membrane of the normal mammary gland, namely LN-1 ($\alpha 1 \beta 1 \gamma 1$), LN-5 ($\alpha 3 \beta 3 \gamma 2$) and LN-10 ($\alpha 5 \beta 1 \gamma 1$) (16). Whilst the role of LNs in cancer progression has been extensively studied, most investigations addressing their role in breast cancer have focussed on LN-1 and LN-5. These studies have established that the expression of both of these isoforms is down-regulated in the majority of invasive breast tumors and that their gradual loss during breast cancer progression contributes to the disruption of basement membrane integrity and loss of epithelial polarity (17-20)

Studies examining LN-10 expression in human breast tumors are limited but variable levels of LN $\alpha 5$ chain have been detected by immunohistochemistry (IHC) in fibroadenoma, ductal carcinoma *in situ* lobular carcinomas, tubular carcinomas, atypical medullary carcinomas and carcinomas of no specific type (18,21). Importantly, the persistence of LN-10 in the vasculature of invasive carcinomas led the authors to conclude that this BM protein may contribute to the attachment of tumor cells to the blood vessel walls during metastasis (21). This is consistent with a recent study by Fujita et al., (22) showing that the angiogenic response associated with breast cancer progression is characterized by a switch in vascular LN isoform expression from LN-9/11 to LN-8/10.

To validate our microarray data and confirm the high level of LN $\alpha 5$ chain expression in bone metastatic 4T1.2 tumors at the protein level, we first compared the expression of LN $\alpha 5$ chains in bone metastatic (4T1.2), weakly metastatic (66cl4) and non-metastatic (67NR) lines of our model by standard IHC performed on primary tumor cryosections (Figure 1). Staining was moderate in 67NR and largely confined to the edge of tumor nests forming a discontinuous BM-like region. In contrast, 66cl4 and 4T1.2 tumors grew disorganized without evidence of a BM. LN $\alpha 5$ was detected in most tumor cells (cytoplasmic and peri-cellular) but was significantly more abundant in highly metastatic 4T1.2 tumors. LN $\alpha 5$ was also abundant in the vasculature.

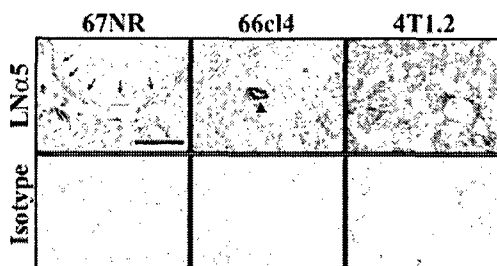


Fig.1. IHC detection of LN $\alpha 5$ chain in primary tumors derived from non-metastatic (67NR), weakly metastatic (66cl4) and highly metastatic (4T1.2) mammary tumor cell lines. Note the BM-like distribution of LN $\alpha 5$ at the periphery of non-metastatic colonies (arrows) and the more widespread cellular expression of this chain in metastatic tumors (66cl4 and 4T1.2) with significantly higher levels in 4T1.2 tumors. LN $\alpha 5$ is also abundant in all blood vessels (arrowhead). Only low non-specific reactivity was detected with a pre-immune IgG control antibody. Scale bar = 50 μ m.

The expression of LN α 5 was also examined in an invasive human breast carcinoma and compared to its expression in adjacent normal breast tissue from the same patient (Figure 2). As expected, LN α 5 localized to the epithelial BM of the normal mammary glands and in surrounding blood vessels as previously reported (16). Strong but more diffuse expression of LN α 5 was detected in the infiltrating tumor cells of the breast carcinoma. Thus, high expression of this LN chain is also a characteristic of some human breast cancers.

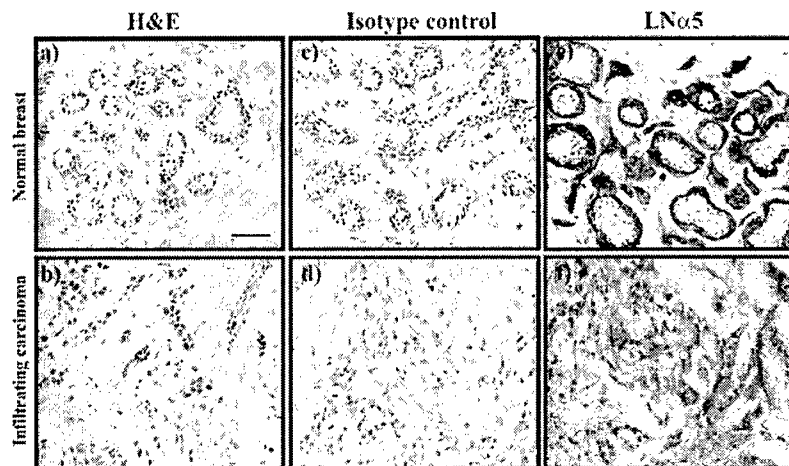


Fig.2. IHC detection of LN α 5 in cryosections of normal and neoplastic human breast tissue. Human breast samples were cryoembedded and the sections stained by standard H&E (a, b) or reacted with isotype control mouse antibody (c, d) or the anti-LN α 5 4C7 antibody (e, f). Note the strong BM reactivity in normal mammary glands (e) and more diffused

expression in breast carcinoma (f). No specific reactivity was detected using a control isotype-matched antibody (c, d). Scale bar: 50 μ m.

Next we set out to compare the expression of LN α 5 to that of LN α 1 (LN-1) and LN α 3 (LN-5) in 4T1.2 primary tumors and spontaneous metastases. However, IHC detection of LN chains in metastases, particularly to bone, has been problematic due to the difficulty of obtaining well preserved bones cryosections and the lack of reactivity of most LN antibodies in standard formalin-fixed paraffin-embedded tissues. To circumvent these problems, we developed a new IHC protocol employing zinc fixation and enzymatic antigen retrieval enabling detection of laminin chains in paraffin-embedded tissues using antibodies previously limited to IHC on cryosections.

As shown in Figure 3, 4T1.2 primary tumors (left column) did not express LN α 1 or LN α 3 but expressed high levels of LN α 5. In lung metastases (middle-left column), LN α 1 was restricted to small areas of the tumor nodule but absent in the lung stroma. LN α 3 was abundant in the lung tissue as reported by others (23,24), but absent in the 4T1.2 tumor nodule. In contrast, strong LN α 5 expression was detected in both 4T1.2 metastatic nodule and surrounding lung stroma. In femur (middle-right column) and spine (right column) metastases, LN α 5 but not LN α 1 or LN α 3 was expressed strongly in all 4T1.2 metastatic cells. Taken together, these results indicate that aggressive 4T1.2 tumors express high levels of LN-10 but not LN-1 or LN-5 and that this phenotype is maintained at metastatic sites. The complete absence of LN-1 and LN-5 in bone metastases contrast with the high expression of LN α 5 and further supports the relevance of LN-10 as a potential prognostic factor for breast cancer.

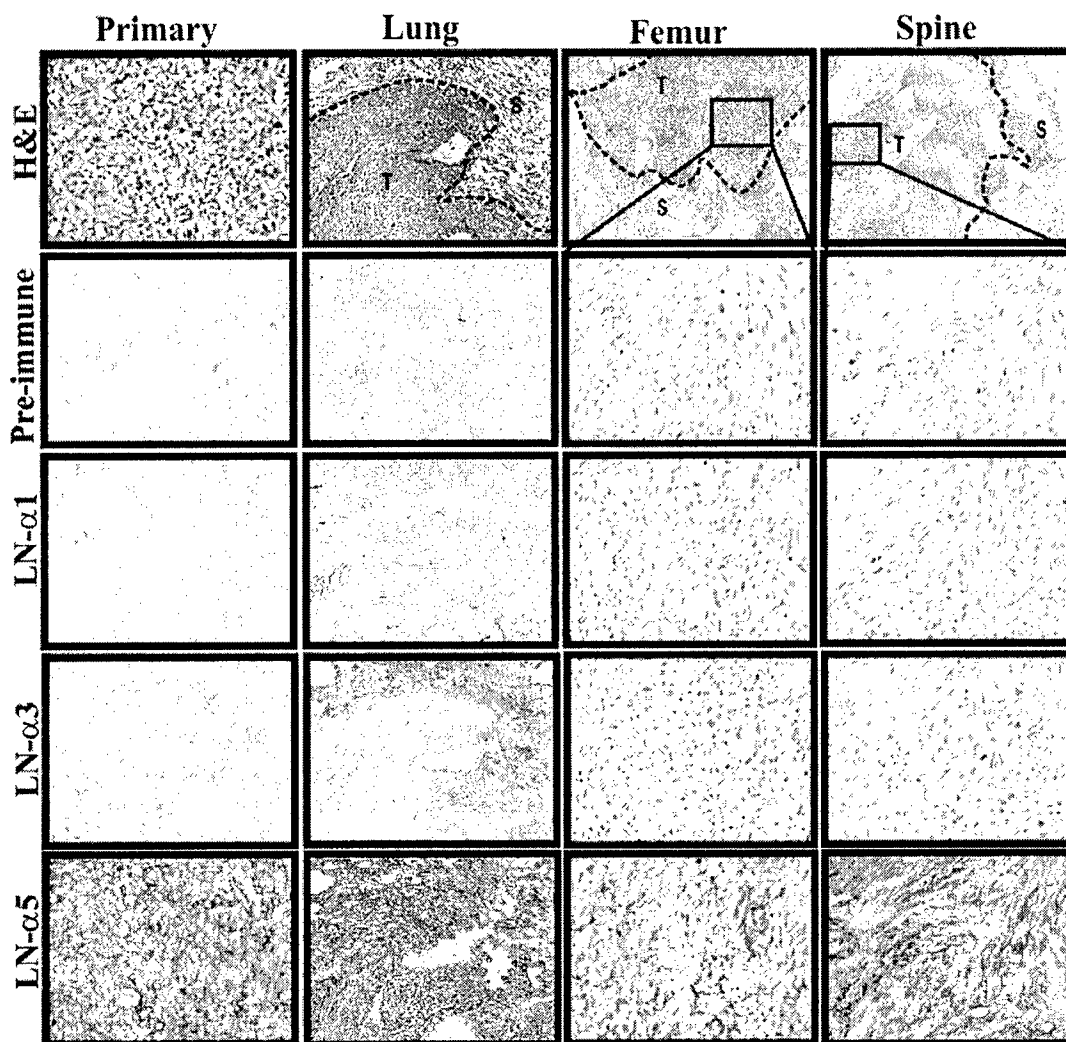


Fig. 3. IHC detection of LN α chains in zinc-fixed, paraffin-embedded 4T1.2 primary tumors and metastases. Zinc-fixed paraffin sections of 4T1.2 primary tumors (left column), lung (middle-left column), femur (middle-right column) and spine (right column) metastases were digested with trypsin (1mg/ml) for 5min at 37°C and stained with H&E (top row) or reacted with rabbit polyclonal antibodies directed against LN α 1, LN α 3 or LN α 5 as indicated. Dotted lines mark the tumor-stroma interface at metastatic sites. Note the persistence of high LN α 5 expression in 4T1.2 lung and bone metastases. No specific reactivity was detected using control pre-immune rabbit antiserum.

b) investigate the adhesive and migratory responses of mammary carcinoma lines to LN-10 and the role of $\alpha v \beta 3$ integrin on these responses

Having confirmed the expression of LN $\alpha 5$ protein in our mammary carcinomas and demonstrated the positive correlation between the level of LN $\alpha 5$ expression and metastatic potential, we addressed the functional relevance of our findings by testing the ability of purified LN-10 to promote adhesion of non-metastatic 67NR cells, weakly metastatic 66cl4 cells and bone metastatic 4T1.2 cells in standard *in vitro* adhesion assays as described previously (25).

Short term adhesion (30min) of 67NR, 66cl4 and 4T1.2 cells was measured in culture plates precoated with BSA, collagen-I (coll-I), collagen-IV (coll-IV), fibronectin (FN), vitronectin (VN) and LN-10 (Figure 4). None of the lines adhered to BSA or collagen-I in this short term assay. Adhesion of all tumor lines was low on fibronectin (<15%). Adhesion to collagen-IV was low but increased with increased metastatic potential of the tumor lines (67NR<66cl4<4T1.2). Similarly, 4T1.2 adhered significantly better to vitronectin (~33%) than 66cl4 and 67NR (~5%). LN-10 was a more potent adhesive substrate for all tumor lines even when used at 2 μ g/ml and all lines adhered equally well.

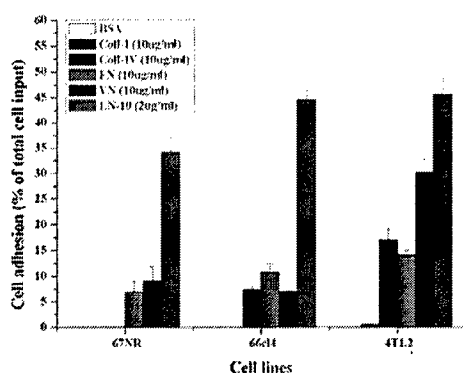


Fig. 4. Adhesion of mammary carcinoma cell lines to ECM substrates. Tumor cells were labeled with calcein and seeded at 2×10^4 /100 μ l serum-free medium in wells precoated with control BSA (1% w/v) or ECM proteins as indicated. After 30min incubation at 37°C, adherent cells were lysed with 1% Triton X-100 and cell adhesion (% of total cell input) determined by measuring the fluorescence in a Molecular FX fluorescent reader. The experiment was repeated twice and the results represent the means \pm SD of triplicates.

To determine the role of $\alpha v \beta 3$ integrin in LN-10-mediated adhesion, the cells were pretreated with function-blocking antibodies directed against the $\beta 3$ integrin subunit and their adhesion to LN-10 and vitronectin tested as described above. A subclone of 66cl4 cells overexpressing $\alpha v \beta 3$ integrin (66cl4 $\beta 3$) was also tested (Figure 5). Adhesion of all tumor lines to LN-10 was not inhibited by anti- $\beta 3$ or control isotype antibodies (left panel). The lack of inhibition was not due to ineffective blocking of $\alpha v \beta 3$ receptor since anti- $\beta 3$ antibodies (but not control antibodies) almost completely prevented adhesion of 4T1.2 and 66cl4 $\beta 3$ cells to vitronectin, a known substrate for this integrin (right panel). As expected, 66cl4 cell adhesion to vitronectin was negligible (~2%), consistent with the lack of $\alpha v \beta 3$ receptors in these cells. We conclude that LN-10 is a potent adhesive substrate for mammary carcinoma cells, irrespective of their metastatic potential and that $\alpha v \beta 3$ integrin is not required for rapid adhesion to this substrate.

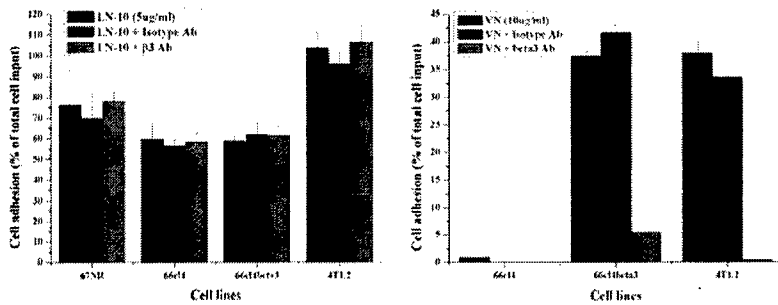


Fig.5. Effect on anti-β3 integrin antibodies on the adhesion of mammary carcinoma cells to LN-10 and vitronectin. The cells were pretreated with anti-β3 or isotype control antibodies for 30min on ice and their adhesion to LN-10 or vitronectin (VN) measured as described in Fig.4.

LN-10 is a migratory substrate for a variety of epithelial and endothelial cell lines in vitro (9,10) (26,27). However, its pro-migratory effect on breast epithelial cells has not been tested. To address whether LN-10 also promotes migration of mammary carcinoma cells of our model, we compared their migratory response to LN-10 in Transwell™ assays. For these assays, LN-10 was coated onto the upper surface of the porous membrane and the cells ($2 \times 10^5/100\mu\text{l}$) seeded in serum-free medium directly on LN-10 coated membrane and allowed to migrate towards medium supplemented with 5% serum in the bottom chamber for 4h at 37°C (Figure 6, left panel). To assess the role of $\alpha\text{v}\beta 3$ integrin on LN-10-mediated chemotactic migration of bone metastatic lines (4T1.2 and 66cl4b3) the cells were pretreated with blocking antibodies against β3 subunit prior to seeding into the chamber wells.

In contrast, to the results obtained from the adhesion assay, LN-10 promoted the migration of 4T1.2 cells (expressing $\alpha\text{v}\beta 3$) but not 67NR or 66cl4 cells and this activity was almost completely inhibited by treatment of the cells with β3-function blocking antibodies (Figure 6, right panel). The 67NR and 66cl4 lines do not express $\alpha\text{v}\beta 3$ integrin. Interestingly, re-expression of $\alpha\text{v}\beta 3$ in weakly metastatic 66cl4 cells (66cl4b3) resulted in a strong increase in migration on LN-10 that was significantly inhibited by anti-β3 antibodies but not by control isotype antibodies. Taken together, these results indicate that LN-10 is a relevant physiological substrate for mammary carcinoma cells and that migration, but not adhesion of highly metastatic tumor cells on LN-10 is dependent, at least in part, on the expression of active $\alpha\text{v}\beta 3$ receptors. The receptor(s) mediating rapid adhesion to LN-10 and the contribution of other integrin receptors in LN-10-mediated migration remain to be determined.

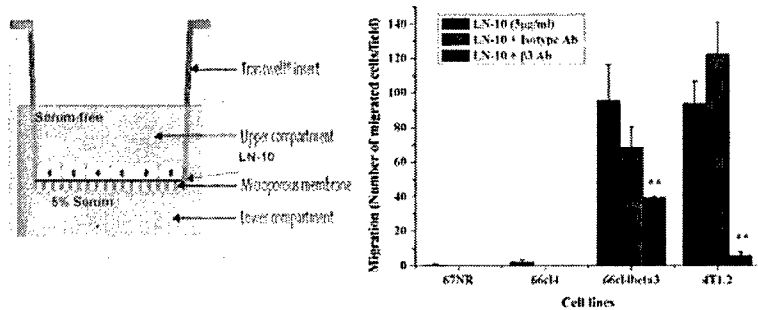


Fig. 6. Integrin $\alpha\text{v}\beta 3$ -dependent chemotactic migration of bone metastatic mammary carcinoma cells on LN-10. The cells were seeded in serum-free medium into Tranwell migration chambers pre-coated with LN-10 (5µg/ml) and their chemotactic migration towards serum-containing medium (5%)

in the bottom chamber measured after 4h at 37°C. Where indicated, the cells were pretreated with control or $\beta 3$ integrin-blocking antibodies (10 μ g/ml) for 30min on ice and added with the cells to the culture wells. After 4h at 37°C, migrated cells attached to the underside of the porous membrane were fixed with formalin, stained with DAPI and counted under a fluorescent microscope. The experiment was repeated twice and the results represent the means \pm SD of triplicates. ** $p < 0.01$.

LN-10 is present in the stroma of common metastatic sites for breast tumors such as the lung and bone (24,28,29). Moreover, LN-10 is abundant in endothelial basement membranes and its vascular expression has been reported to increase during breast tumor progression (18,21,22) leading to the suggestion that vascular LN-10 may facilitate tumor cell attachment and migration during vascular dissemination of breast tumors. Based on these observations we asked whether LN-10 was sufficient alone to promote haptotactic migration of 66cl4, 66cl4 $\beta 3$ and 4T1.2 tumor cells. To test this, we performed Transwell migration assays by precoating the underside of the porous membrane with LN-10 and measured transmigration of tumor cells towards LN-10 in the absence of serum (Figure 7, left panel).

As shown in Figure 7 (right panel), 66cl4 cells migrated efficiently in this assay despite the lack of $\alpha v \beta 3$ integrin expression in these cells. 4T1.2 and 66cl4 $\beta 3$ cells also migrated towards LN-10 but unlike the chemotactic response, haptotaxis was not inhibited significantly by anti- $\beta 3$ (or isotype control antibodies). These results strongly suggest that LN-10 is a potent haptotactic factor for breast tumor cells. However, the haptotactic response can be distinguished from chemotactic migration on LN-10 by the specific receptor(s) used in these responses. We are currently investigating the LN-10 receptor(s) involved in the haptotactic response. A likely candidate include $\alpha 3 \beta 1$ integrin, a major LN receptor that has been reported to mediate haptotaxis of MDA-MD-231 breast carcinoma cells towards LN-1 and to regulate expression of MMP-9 in these cells (30).

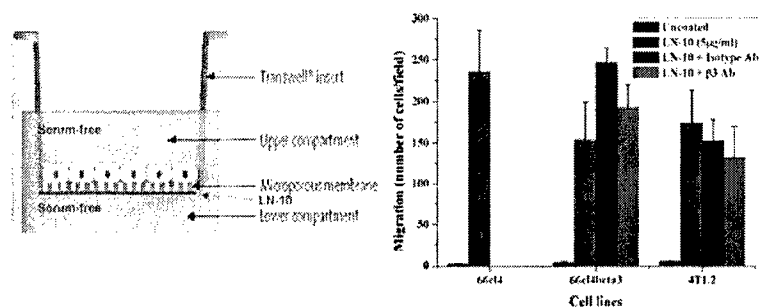


Fig.7. LN-10-mediated haptotactic migration of mammary carcinoma cells is $\alpha v \beta 3$ integrin-independent. The underside of the Transwell porous membrane was coated with LN-10 (5 μ g/ml) and the cells ($2 \times 10^5/100\mu$ l) seeded in serum-free medium in the upper well. Haptotactic migration towards LN-10 in the absence of serum in the bottom chamber was measured after 4h at 37°C. Where indicated, the cells were pretreated with control or $\beta 3$ integrin-blocking antibodies (10 μ g/ml). The experiment was repeated twice and the results represent the means \pm SD of triplicates.

Task 2. Determine the involvement of MMP-9 in LN-10-mediated migration

Matrix metalloproteinases (MMP) are proteolytic enzymes whose role in tumor progression has been extensively documented (31). In particular, tumor and stromal expression and activity of the gelatinases MMP-2 and MMP-9 is often upregulated in breast tumors (32-35) and is thought to contribute to basement membrane dissolution seen during breast cancer progression and the acquisition of motile and invasive properties by breast tumor cells. Interestingly, many studies have shown that cellular release of gelatinases is regulated by ECM-receptor interactions. This induction appears to be cell type-specific and most likely dictated by substrate availability and the specific receptor(s) expressed in a given cell type. For instance, vitronectin stimulates the release of active MMP-9 in lymphoid cells through engagement of $\alpha v \beta 3$ integrins (36). In contrast, increased secretion of mature MMP-9 by MDA-MB-435 breast tumor cells occurs following $\alpha v \beta 3$ -dependent attachment to fibrinogen and to a lesser extent to fibronectin but not to vitronectin (7). Cooperation between active $\alpha v \beta 3$ and mature MMP-9 in turn enhances migration of breast cancer cells, a response likely to contribute to the greater propensity of breast cancer cells expressing active $\alpha v \beta 3$ integrin to metastasize to bone (37).

LN-10s also regulate expression and release of gelatinases. MDA-MB-231 breast tumor cell invasion and haptotactic migration towards LN-10 is accompanied by the release of higher levels of MMP-9 gelatinase activity and these responses are abrogated by treatment with antibodies blocking the $\alpha 3 \beta 1$ integrin receptor (30). Whilst induction of MMP-2 and MMP-9 activity in response to LN-10 has not been reported in breast epithelial cells, studies in other cell types strongly suggest that LN-10 is likely to play a similar role in mammary carcinoma cells. For instance, adhesion of A549 lung carcinoma cells to a commercial preparation of LN-10/11 (or to LN-5) was shown to stimulate secretion of mature MMP-2 (but not MMP-9) in culture via $\alpha 3 \beta 1$ integrin-mediated attachment (38). MMP-2 appeared to contribute to LN-10/11-mediated migration in these cells since this response was partially suppressed by treatment with a gelatinase inhibitor. Others have reported that the AQARSAAKVKVSMKF peptide derived from the $\alpha 5$ chain of LN-10 but not the corresponding peptide derived from the $\alpha 3$ chain of LN-5 is a potent inducer of MMP-9 (but not MMP-2) expression in macrophages *in vitro* and *in vivo* (12,39).

We have reported that the 4T1.2 mammary carcinoma line secretes significantly higher levels of MMP-9 in culture than weakly 66cl4 and non-metastatic 67NR cells (40). None of these lines express detectable levels of MMP-2. Given the cooperative role of MMP-9 and $\alpha v \beta 3$ in breast cancer metastasis to bone reported by others and our demonstration that LN-10 selectively promotes chemotactic migration of $\alpha v \beta 3$ -expressing bone metastatic lines (4T1.2 and 66cl4 $\beta 3$), it was of interest to determine whether LN-10 could induce secretion of MMP-9 in the tumor lines of our model.

For these experiments, 67NR, 66cl4 and 4T1.2 cells were seeded in culture plates coated with BSA, collagen-I, collagen-IV or LN-10 and incubated for 42h at 37°C in serum-free medium. The culture supernatants were then analyzed for gelatinolytic activity by gelatin zymography (Figure 8). As expected control 4T1.2 cultures seeded on BSA secreted significantly higher levels of MMP-9 activity than 67NR and 66cl4. MMP-9 expression remained low in 67NR and 66cl4 cells irrespective of the substrate used. In contrast, MMP-9 activity detected in culture supernatants of 4T1.2 cells seeded on collagen-I and collagen-IV was marginally lower but increased 2.5-fold when the cells were seeded on LN-10. We are currently investigating whether $\alpha v \beta 3$ integrin receptors are required for this response using anti- $\beta 3$ blocking antibodies to inhibit the release of MMP-9 activity.

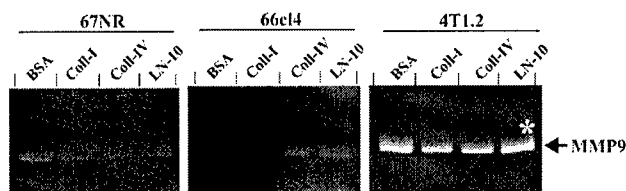


Fig. 8. Induction of MMP-9 activity in mammary carcinoma cells by ECM substrates. Tumor cells were seeded in BSA (2%w/v), collagen-I (20 μ g/ml), collagen-IV (20 μ g/ml) or LN-10 (5 μ g/ml)-coated plates and incubated for 42h at 37°C in serum-free medium.

Culture supernatants were then analyzed for gelatinolytic activity by standard gelatin zymography.

To further investigate the role of MMP-9 in LN-10-mediated adhesion and migration, we derived subclones of 4T1.2 cells with reduced MMP-9 expression by RNA interference. Retroviral vectors (pRetroSuper, pRs) encoding a puromycin-resistance gene and specific short hairpin RNAs targeting MMP-9 (or green fluorescence protein as control) were transfected into the Phoenix packaging line and the retroviral supernatants used to infect 4T1.2 cells. Following selection of infected cells in the presence of puromycin, single cell clones were selected for reduced MMP-9 expression by immunoblot and gelatin zymography (Figure 9). Five control 4T1.2pRs-GFP clones showed MMP-9 protein expression by immunoblot and activity by zymography (left panels) comparable to that seen in the parental 4T1.2 line (not shown). In contrast, five 4T1.2pRs-MMP-9 clones had no detectable MMP-9 proteins and displayed only modest or no detectable MMP-9 activity by zymography (right panels). Individual clones from respective groups were pooled for further experiments.

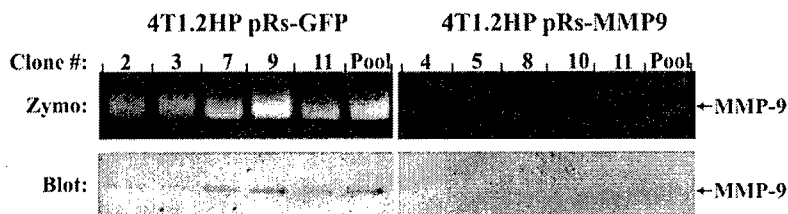


Fig. 9. Silencing of MMP-9 in 4T1.2 cells by RNA inhibition. Short hairpin RNA constructs targeting MMP-9 and GFP (control) were introduced into 4T1.2 cell by retroviral infection using a pRetroSuper vector encoding a puromycin resistance gene. Infected cells were selected in the presence of puromycin and individual clones tested for MMP-9 protein expression by immunoblot (bottom panels) and activity by gelatin zymography (top panels). The figure shows 5 individual clones and pool for control (4T1.2pRs-GFP) and MMP-9-deficient (4T1.2pRs-MMP9) cells.

Adhesion of 4T1.2pRs-GFP and 4T1.2pRs-MMP-9 pools to LN-10 was tested in short term adhesion assays in the presence or absence of anti- β 3 integrin an isotype control antibodies (Figure 10). As expected, control 4T1.2pRs-GFP cells adhered strongly to LN-10 and adhesion was not inhibited by anti- β 3 or isotype antibodies. Similarly, anti- β 3 antibodies did not block adhesion of 4T1.2pRs-MMP9 cells to LN-10 and their adhesion was only marginally higher than control cells (not statistically significant). It is noteworthy however that 4T1.2pRs-MMP9 cells consistently appeared more flattened in culture. This effect was evident even after 48h in culture and independent of the substrate used suggesting a less motile phenotype (data not shown).

To test this hypothesis, migration of 4T1.2pRs-GFP and 4T1.2pRs-MMP9 cells was compared in the chemotactic Transwell assay (Figure 11). Neither cell lines migrated significantly in the absence of serum in the bottom chamber. Whereas LN-10 alone was insufficient to promote migration of control 4T1.2pRs-GFP cells across the porous membrane, it enhanced their chemotactic response to serum (~60 versus 100 cells/field). Importantly, MMP-9 silencing in 4T1.2pRs-MMP9 significantly impaired

chemotactic migration both in the presence and absence of LN-10 compared to control cells. Taken together, these results indicate that that MMP-9 activity contributes to chemotactic migration of 4T1.2 cells. Since MMP-9 silencing inhibited chemotactic migration on LN-10 to a similar extent as that observed with anti- $\beta 3$ integrin antibodies (see Figure 6), we speculate that engagement of $\alpha v \beta 3$ integrin by LN-10 may regulate the activity or release of MMP-9 in 4T1.2 cells. This is being addressed by testing the effect of anti- $\beta 3$ blocking antibodies on LN-10-mediated induction of MMP-9 activity in 4T1.2 cells.

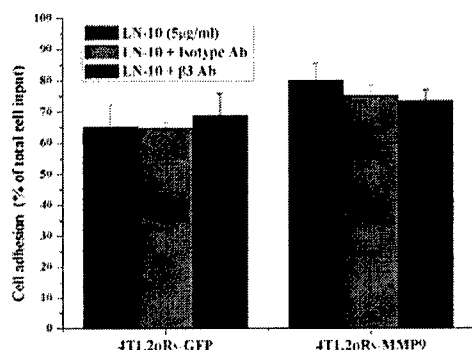


Fig. 10. Effect of MMP-9 silencing on adhesion of bone metastatic 4T1.2 cells to LN-10. Pools of control 4T1.2 subclones expressing MMP-9 (4T1.2pRs-GFP) and subclones with reduced expression of MMP-9 (4T1.2pRs-MMP9) were tested for adhesion to LN-10 as described in the legend of Figure 4. The experiment was repeated twice and the results represent the means \pm SD of triplicates.

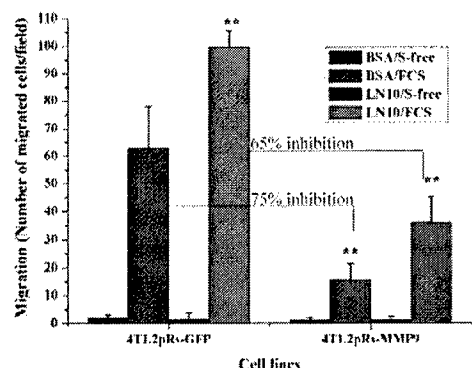


Fig. 11. Effect of MMP-9 silencing on LN-10-mediated chemotactic migration of mammary carcinoma cells. Migration of control 4T1.2pRs-GFP and 4T1.2pRs-MMP9 knockdown pool of cells was compared in the chemotaxis assay as described in the legend of Figure 6. Where indicated, LN-10 (5µg/ml) or BSA (1%) were precoated onto the upper surface of the Transwell porous membrane and serum (5%) was added to the bottom chamber. **p < 0.01.

The effect of MMP-9 down-regulation was also tested on haptotactic response to LN-10 in the absence of serum. As shown in Figure 12, neither 4T1.2pRs-GFP nor 4T1.2pRs-MMP9 migrated through the Transwell membrane in the absence of LN-10 coating. In stark contrast to the chemotactic responses however, both tumour lines migrated strongly towards LN-10. These results indicate that haptotactic migration towards LN-10 does not require MMP-9 activity. This conclusion is consistent also with the possibility that the chemotactic and haptotactic responses of mammary carcinoma cells to LN-10 are mediated through different receptors and signaling pathways.

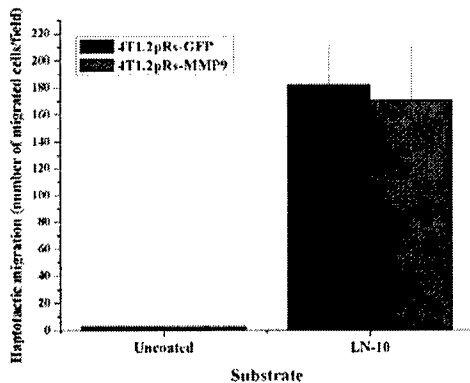


Fig. 12. Effect of MMP-9 silencing on LN-10-mediated haptotactic migration of mammary carcinoma cells. Migration of control 4T1.2pRs-GFP and 4T1.2pRs-MMP9 knockdown pool of cells was compared in the haptotactic assay as described in the legend of Figure 7. For this assay, the underside of the Transwell porous membrane was left untreated or coated with LN-10 (5 μ g/ml) in the absence of serum in the upper and bottom chambers.

Task 3. Provide evidence for specific interactions between tumor $\alpha v \beta 3$ integrin and endothelial LN-10 during intra/extravasation process

For successful metastasis, tumor cells must first invade and migrate through the surrounding stroma, attach to the endothelium and intravasate into the vasculature. Binding of breast carcinoma cells to the endothelium is dependent on integrin-mediated attachment to the subendothelial matrix (41). Thus the abundance of LN-10 but not LN-1 or LN-5 in blood vessels and increased vascular expression of LN-10 recently reported in invasive breast carcinoma is consistent with the potential role of LN-10 in mediating vascular dissemination of tumor cells (18,21,22). Moreover, the presence of LN-10 in the sinusoids of the bone through which tumor cells must extravasate to enter the bone environment (42) contrasts with the complete absence of LN-1 and LN-5 (28) and further supports the relevance of LN-10 in the metastasis of breast tumors to bone.

To provide evidence the role of endothelial LN-10 in intra/extravasation of breast tumor cells, we first developed an *in vitro* assay measuring the ability of mammary carcinoma cells to interact with and migrate through a monolayer of endothelial cells. Mouse microvascular endothelial cells (bEnd.3) were seeded in the upper well of Transwell migration chambers and allowed to form a confluent monolayer overnight at 37°C. Excess cells and medium were removed and calcein-labeled tumor cells added to the upper chamber in serum-free medium. Serum-containing medium was then added to the bottom chamber as chemoattractant and the cells incubated for a further 18h to allow chemotactic migration of tumor cells through the endothelial monolayer and porous membrane. Tumour cells were also seeded in the absence of endothelial cells to verify that calcein labeling does not impair their ability to migrate. Calcein labeling did not affect tumor cell viability (as judged by trypan blue staining) or their ability to migrate in standard chemotactic assays (black bars). As expected, the bone metastatic lines 4T1.2 and 4T1.13 but not 66cl4 or 67NR migrated towards the gradient of serum in the absence of endothelial cell monolayer. Similarly, only the bone metastatic lines migrated through the endothelial monolayer albeit at a lower rate (red bars). Transendothelial migration also correlated well with the respective bone metastatic potential of 4T1.2 and 4T1.13 *in vivo*.

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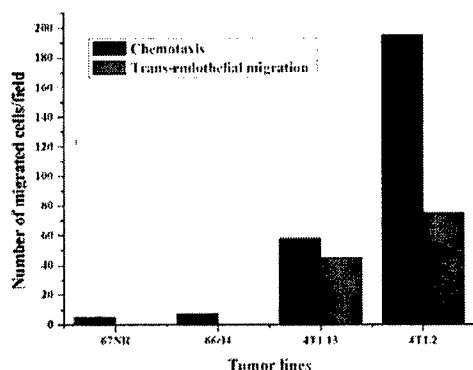


Fig. 13. Transendothelial migration of mammary carcinoma cells. Mouse microvascular endothelial cells (bEnd.3, 1×10^5) were seeded in complete medium in the upper well of Transwells and allowed to form a confluent monolayer on the surface of the porous membrane overnight at 37°C. Unattached cells and medium were removed and calcein-labeled tumor cells ($2 \times 10^5/200\mu\text{l}$ in serum-free medium) added to the upper well. 600 μl of medium supplemented with 5% serum was added to the bottom wells as chemoattractant. After 18h incubation at 37°C, cells in the upper chamber were removed with a cotton swab and migrated cells on

the underside of the membrane fixed in formalin and stained with DAPI. Transendothelial migration (red bars) was visualized under a fluorescence microscope and only tumor cells with a green cytoplasm (calcein) and blue nucleus (DAPI) were counted. As control, tumor cells only were added to the upper wells for chemotactic migration (black bars). The results show the number of migrated cells from one representative experiment.

To specifically address the role of endothelial LN-10 in tumor trans-endothelial migration, we proposed to down-regulate the expression of LN α 5 chain in bEnd.3 cells using the same strategy successfully employed for down-regulation of MMP-9 (see Figure 9). Progress with these experiments is summarized in Table 1 below. Over 60 target sequences distributed over the entire LN- α 5 gene that meet all criteria for optimal siRNA design have been identified and three LN- α 5 sequences and corresponding scrambled sequences were selected. We have so far successfully prepared retroviral vectors targeting the LN α 5 chain (pRs-LAMA5) and corresponding scrambled vector (pRs-LAMA5S) and infected bEnd.3 and 4T1.2 tumor cells. The rationale for down-regulating LN α 5 in 4T1.2 cells is that these cells are easily infected with the pRetroSuper vector and their rapid growth rate permits faster selection and expansion of infected clones for validation of LN α 5 silencing. Preliminary screening of 21 individual 4T1.2pRs-LAMA5 clones by real-time quantitative RT-PCR has identified 13 clones with variable levels of LN α 5 silencing (Figure 14).

The LN α 5 relative transcript abundance (RTA, relative to GAPDH expression) for each clone was compared to that of the parental 4T1.2 line and the results expressed as % LN α 5 RTA of parental 4T1.2 cells. Of the 13 clones with reduced expression, 5 clones with less than 30% of LN α 5 expression in 4T1.2 cells were selected (asterisks). Down-regulation of protein expression will be confirmed by immunocytochemistry. The same strategy will be used to confirm down-regulation of LN α 5 in bEnd.3pRs-LAMA5 (or lack of down-regulation in bEnd.3LAMA5S bulk cultures) prior to commencing trans-endothelial migration studies. We anticipate this work to be completed by September 2005.

Table 1. Development of bEnd.3 LAMA5 and LAMA5-Scrambled siRNA cell lines for trans-endothelial migration

Tasks	LAMA5 siRNA	LAMA5-Scrambled siRNA
Identify suitable sequences from the mouse LN α 5 mRNA for RNA inhibition	Completed	Completed
Construction of LAMA5 and Scrambled retroviral vectors	Completed	Completed
Transfection of Phoenix packaging line	Completed	Completed
Infection of cell lines with retroviral supernatant	Completed for 4T1.2 tumor cells and bEnd.3 endothelial cells	Completed for 4T1.2 tumor cells and bEnd.3 endothelial cells
Puromycin selection of bulk cultures	Completed for 4T1.2 and bEnd.3 cells	Completed for 4T1.2 and bEnd.3 cells
Isolation and expansion of single cell clones	Completed for 4T1.2 cells Initiated for bEnd.3 cells	Only bulk cultures to be used for 4T1.2 and bEnd.3 cells
Verification of mRNA down-regulation by real-time quantitative RT-PCR	Completed for 4T1.2 cells (Fig. 14) Expression confirmed in parental bEnd.3 and screening underway for subclones	Underway for 4T1.2 and bEnd.3 bulk cultures
Confirmation of protein down-regulation by immunocytochemistry	Underway for 4T1.2 subclones	Expected completion (Aug. 2005)
Trans-endothelial migration assays in vitro	Expected completion (Sept. 2005)	Expected completion (Sept. 2005)

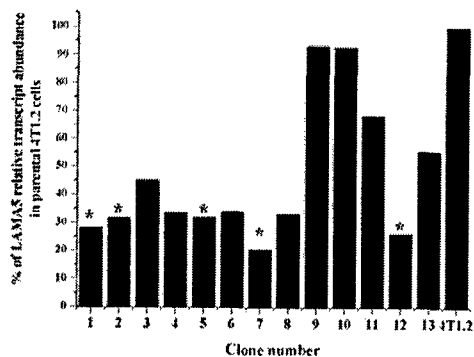


Fig. 14. Down-regulation of LN α 5 mRNA expression by siRNA. 4T1.2 cells were infected with a retroviral vector targeting LN α 5 (4T1.2pRs-LAMA5) and infected cells selected in the presence of puromycin. Bulk cultures of puromycin-resistant cells were single cell cloned and the relative LN α 5 transcript abundance (RTA, relative to GAPDH) compared to LN α 5 RTA in parental 4T1.2 cells. The results show 13 clones with reduced LN α 5 mRNA expression. Five clones with $\geq 70\%$ decrease in LN α 5 RTA were selected for confirmation of LN α 5 protein down-regulation in vivo (*).

Task 4. Screen LN-10 peptides for their migration inhibitory activity *in vitro* and test the effect of selected LN-10 peptides for their ability to suppress breast cancer metastasis to bone *in vivo*.

A large number of LN fragments or bioactive peptides have been isolated and shown to possess stimulatory or inhibitory activity on tumor cells *in vitro* and in experimental models (43-45). For instance, the YIGSR sequence found in the LN α 1 chain (present in several isoforms including LN-10) was reported to inhibit osteolytic metastases following intracardiac inoculation of melanoma cells in nude mice (46). Conversely, the integrin α v β 3 and α 5 β 1-binding C16 peptide derived from the LN γ 1 chain enhances melanoma pulmonary metastasis in an experimental model (47). Antagonists of this peptide (C16Y and C16S) were reported to possess potent anti-angiogenic effects and to inhibit tumor growth following subcutaneous injection of MDA-231 breast cancer cells in nude mice without inhibiting their proliferation *in vitro* (48).

More recently, evidence for specific sequences of the LN α 5 chain mediating various biological activities has emerged (11,12,49,50). In particular, screening of a large number of overlapping peptides from the LN- α 5 N-terminal domain VI and C-terminal globular domain for cell attachment activity has led to the identification of a novel receptor for LN-10 (CD44) and of at least 4 peptides with anti-tumor growth and metastatic activity against B16-F10 melanoma cells (11,49).

In collaboration with Dr. Hynda Kleinman (NIH), we have initiated studies to identify potential peptide inhibitors of LN-10 functions in breast tumor cells. Many of these peptides have been shown to mediate cell adhesion *in vitro* and/or to inhibit angiogenesis, tumor growth and metastasis *in vivo* (Table 2). Of particular interest were two peptides derived from the LN γ 1 chain (C16Y) and α 5 chain (A5A13b) known to interact with α v β 3 integrin receptor.

Table 2. LN-10 peptides to be tested for inhibitory activity on cell migration *in vitro* spontaneous metastasis *in vivo*

Peptide	LN chain	Comments/Reported activity	Receptor	Reference
C16Y	LN γ 1	Blocks attachment to LN-1 Anti-tumor/anti-angiogenic	α v β 3, α 5 β 1	(48,51)
C16YS	-	Control Scrambled C16Y	-	(48,51)
A13b	LN α 1 Domain VI	Strong cell attachment. Truncated version of A13 mediating migration, angiogenesis and metastasis	α 5 β 1, α v β 3	(49,51,52)
A5(A13b)	LN α 5 Domain VI	Homologue of A13b Strong cell attachment	Most likely α 5 β 1 α v β 3	(49)
A5G27	LN α 5 G- domain	Inhibits cell migration, invasion of Matrigel, angiogenesis and lung colonization	CD44	(11)
A5G73	LN α 5 G- domain	Moderate cell attachment Inhibits lung colonization	?	(11)

These peptides were tested for their inhibitory activity on the chemotactic migration of 4T1.2 cells on LN-10 (Figure 15). 4T1.2 cells were left untreated or treated with increasing concentrations of peptides on ice for 20min and added to the Transwells. Control untreated cells migrated efficiently and

migration was not inhibited by the A5G73 peptide. In contrast, the C16Y peptide but not the scrambled version (C16YS) inhibited chemotactic migration in a dose-dependent manner. The LN α 5 peptide A5A13b and the corresponding peptide derived from the LN α 1 chain (A13b) inhibited migration by more than 50% when used at 25 μ g/ml. Another peptide derived from the globular domain of LN α 5 chain and known to bind CD44 receptor (A5G27) inhibited 4T1.2 cell migration the most in this assay at 25 μ g/ml resulting in ~85% inhibition. CD44 has been implicated in breast cancer progression (53) and was shown to be up-regulated in MDA-MB-231 in response to hepatocyte growth factor and mediate adhesion to endothelial cells and trans-endothelial migration (54). We have confirmed the expression of CD44 in all tumor cell lines of the model and are currently investigating the specific variants expressed in bone metastatic lines. A5G27 and a scrambled version of this peptide are currently being synthesized in sufficient quantity (400-500mg) for testing in our in vivo model of metastasis. These experiments will be initiated as soon as the peptides are received and we anticipate the work to be completed by Dec 2005.

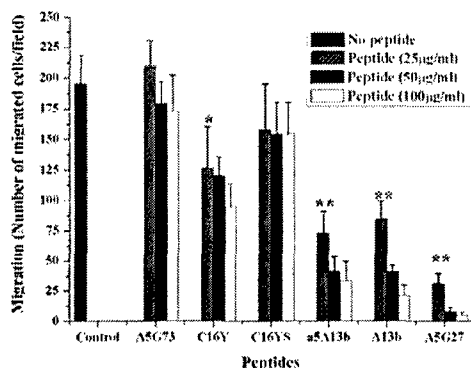


Fig. 15. Effect of inhibitory LN peptides on 4T1.2 chemotactic migration on LN-10. Chemotactic migration assays were performed as described in the legend of Figure 6. 4T1.2 cells were left untreated of pretreated on ice for 20min in the presence of indicated concentrations of peptides and added to the upper well of Transwells pre-coated with LN-10 (5 μ g/ml). Migration towards a gradient of serum (5%) in the bottom well was measured after 4h incubation at 37°C. The experiment was repeated twice and the results represent the means \pm SD of triplicates. * p <0.01, ** p <0.001.

KEY RESEARCH ACCOMPLISHMENTS

- Development of an IHC staining protocol for LN chains in paraffin-embedded tissues
- Demonstration that LN-10 ($\alpha 5$ chain) but not LN-1 ($\alpha 1$ chain) or LN-5 ($\alpha 3$ chain) is more highly expressed in primary breast tumors and metastases derived from bone metastatic mammary carcinoma cells than in weakly and non-metastatic tumors.
- Demonstration that LN-10 is a potent adhesive substrate for mammary carcinoma cells
- Demonstration that LN-10 mediates haptotaxis in the absence of serum and enhances chemotactic migration of breast cancer towards serum
- Demonstration that $\alpha v \beta 3$ integrin selectively mediates chemotactic migration of bone metastatic mammary carcinoma cells on LN-10
- Demonstration that LN-10 promotes the release of MMP-9 in bone metastatic mammary carcinoma cells
- Demonstration that MMP-9 is required for chemotactic migration of mammary carcinoma cells on LN-10 but not for haptotactic migration towards LN-10
- Identification of LN peptides with potent anti-migratory activity on mammary carcinoma cells on LN-10
- Development of mammary tumor and endothelial cell variants with reduced LN $\alpha 5$ expression by siRNA

REPORTABLE OUTCOMES

Invited speaker:

Laminin-10: expression and functional role in the metastasis of mammary carcinomas. Ludwig Institute for Cancer Research, Parkville, VIC, Australia. April 2005.

Functional interplay between laminin-10, $\alpha v \beta 3$ integrin and MMP-9 in spontaneous metastasis of breast tumors to bone. University of Sherbrooke, Sherbrooke, Qc, Canada. June 2005

Interplay between laminin-10, $\alpha v \beta 3$ integrin and MMP-9 in spontaneous metastasis of breast tumors to bone. National Institute of Health, Bethesda, MD, USA. June 2005

Meeting presentations:

Microenvironmental regulation of breast tumours:evidence for the cooperative role of $\alpha v \beta 3$ integrin, laminin-10 and matrix metalloproteinase-9 in breast cancer metastasis to bone. N. Pouliot, A.L. Natoli, B. Parker, E. Sloan, B. Eckhardt, L. Zamurs, E. Nice and R. Anderson. Poster presentation at the 17th Lorne Cancer Conference, Philip Island, VIC, Australia, June 2005.

Functional interactions between laminin-10, $\alpha v \beta 3$ integrin and MMP-9 in breast cancer metastasis to bone. N. Pouliot, A. Natoli, B. Parker, E. Sloan, L. Zamurs, E. Nice and R. Anderson. Poster presentation at the Era of Hope, Department of Defense Breast Cancer Research Program meeting, Philadelphia, PA, USA, June 2005.

Manuscripts in preparation:

Immunohistochemical detection of laminin α chains in primary mammary tumors and spontaneous bone metastases of a mouse model:evidence for the functional role of laminin-10 in breast cancer metastasis to bone. Chia J., Anderson R., Parker B., Nice E., Zamurs L. and Pouliot N Manuscript in preparation.

LN-10 modulates $\alpha v \beta 3$ integrin and MMP-9-dependent migration of mammary carcinoma cells. Chia J., Natoli. A., Anderson R., Kleinman H., Parker B., Nice E., Zamurs L. and Pouliot N.. Manuscript in preparation.

Funding applications:

Funding has been requested from the National Health & Medical Research Council of Australia and an application to The Susan G. Komen Breast Cancer Foundation will be submitted to continue the research project initiated through this DoD Concept Award #81XWH-04-1-0707.

CONCLUSIONS

Based on evidence from the literature and our findings that LN-10, MMP-9 and the $\alpha v \beta 3$ integrin receptor are more highly expressed in bone metastatic mammary tumors than in weakly and non-metastatic tumors in a syngeneic model of spontaneous breast cancer metastasis, we hypothesized that these three proteins cooperate functionally in breast cancer metastasis to bone. We have developed a new IHC protocol for detection of LN chains in paraffin-embedded tissues and confirmed that LN-10 but not LN-1 or LN-5 is highly expressed in primary breast tumors and metastases, including those in bone. Moreover, we demonstrated that LN-10 is a potent functional adhesive substrate for mammary carcinoma cells, regulating migration, protease secretion and trans-endothelial migration. We found that integrin $\alpha v \beta 3$ is a receptor for LN-10 in bone metastatic tumor cells and mediates chemotactic but not haptotactic migration on LN-10. Furthermore, MMP-9 appears to be required for chemotactic migration (but not haptotaxis) as migration was dramatically decreased in this assay by down-regulation of MMP-9 expression in bone metastatic 4T1.2 cells by siRNA.

We have tested the effect of LN peptides on 4T1.2 chemotactic migration and identified two $\alpha v \beta 3$ integrin-binding peptides (A5A13b, $\alpha 5$ chain and C16Y, $\gamma 1$ chain) and one CD44-binding peptide (A5G27, $\alpha 5$ chain) that strongly inhibited LN-10-mediated chemotactic migration. We have generated siRNA constructs to suppress LN $\alpha 5$ expression in 4T1.2 and bEnd.3 endothelial cells. Experiments using inhibitory peptides and siRNA are currently underway to address the role of tumor-derived LN-10 in spontaneous metastasis to bone in vivo and the role of endothelial LN-10 in mediating tumor cell attachment and trans-endothelial migration. Taken together, the results obtained are strongly supportive of our hypothesis that functional cooperation between LN-10, $\alpha v \beta 3$ integrin and MMP-9 contributes to breast cancer metastasis to bone. In addition, our results suggest that LN-10 may be a useful prognostic indicator and/or potential therapeutic target for aggressive breast tumors.

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Nationality: Canadian/Australian

Languages: English and French (spoken and written)

Education:

August 1992 – June 1998	Doctor of Philosophy (Cancer Research) Ludwig Institute for Cancer Research University of Melbourne, Victoria, Australia
September 1995 – October 1995	Leadership and Professional Development Program University of Melbourne School of Graduate Studies/Melbourne Business School Victoria, Australia
September 1989 - July 1991	Master of Science (Immunology) University of Sherbrooke Québec, Canada
January 1986 - April 1989	Bachelor of Science (Biotechnology) University of Québec in Montreal Québec, Canada
September 1984 - December 1985	College Diploma in Science Collège de Montmorency Québec, Canada

Present Appointment:

June 2000 – Current	Research Fellow Peter MacCallum Cancer Centre St-Andrews Place, East Melbourne Victoria, Australia 3002
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Post-Doctoral Fellowships:

July 2004 – June 2005	Concept Award	U.S.Army Medical Research & Materiel Command
April 2003 – June 2004	Post-Doctoral Fellowship	U.S.Army Medical Research & Materiel Command
January 2003 – December 2005	Post-Doctoral Fellowship	NH&MRC Project Grant (Declined)
January 1999 – December 1999	Post-Doctoral Fellowship	Anti-Cancer Council of Victoria (ACCV)

Post-Graduate Scholarships and Awards:

September 1992 - March 1996	Ph.D. Scholarship:	Commonwealth Scholarship and Fellowship Plan (CSFP)
September 1990 - September 1991	M.Sc. Scholarship:	Fonds Canadiens d'Aide à la Recherche (FCAR)
September 1989 - September 1990	M.Sc. Scholarship	Fonds Canadiens d'Aide à la Recherche (FCAR)
June 1989 - August 1989	M.Sc. Excellence Award	Fonds de Recherche en Santé du Québec (FRSQ)
May 1989 - September 1989	B.Sc. Scholarship	Fonds de Recherche en Santé du Québec (FRSQ)
May 1988 - September 1988	B.Sc. Scholarship	Conseil de Recherche en Science Naturelles et en Génie (CRSNG)

Research Experience:**Current Research Project (April 2003 – Current):**

“Breast Tumour/Stromal Cell Interactions in Bone”
Peter MacCallum Cancer Centre

The aim of my current research is to investigate factors that regulate the establishment of breast cancer metastases in bone. Specifically, I am using an orthotopic mouse model of breast cancer metastasis to bone established in Dr. Robin Anderson's lab at Peter MacCallum Cancer Centre which employs a variety of breast cancer cell line that differ in their metastatic potential. Tumor burden in bone and other tissues following injection of tumor lines into the mammary gland is measured using a real time quantitative PCR (RTQ-PCR) method developed in our laboratory. Other approaches to investigate factors involved in bone metastasis include the use of K/O animals ($\beta 3$ integrin, MMP-9, MMP-12 and M-CSF), specific inhibitors or overexpression construct for selected candidate genes and RNA interference technology both in our *in vivo* model and in *in vitro* co-culture assays. Expression of several factors in the tumour/stroma microenvironment is also being investigated by immunohistochemistry and immunofluorescence microscopy with particular emphasis on RANKL, OPG MMPs, integrins and extracellular matrix proteins.

Post-Doctoral Research July 2000 – March 2003):

“Microenvironmental regulation of the tissue regenerative capacity of keratinocyte stem cells and their progeny”
Peter MacCallum Cancer Centre

Project: The general objective of this research project was to explore the functional role of the mesenchymal microenvironment (with particular emphasis on laminin-10/11, LN-10/11) in regulating the tissue regenerative capacity of keratinocyte stem cells. I have demonstrated the presence of LN-10/11 in the cutaneous basement membrane and identified 2 receptors in keratinocytes. Further I have demonstrated that LN-10/11 has a functional role in promoting adhesion, proliferation and migration of normal and tumourigenic keratinocytes *in vitro*. This work has been published in *Exp. Dermatol.* and was later extended to an *in vitro* organotypic skin regeneration model. In this system, I have provided strong evidence that LN-10/11 is an important regulatory molecule promoting skin regeneration in part by delaying the onset of keratinocyte terminal differentiation and recruiting early differentiating keratinocytes into proliferation. This work was recently published in *J. Clin. Invest.*. The role of soluble dermal factors including FGF-7 and FGF-10 and signaling pathways regulating the expression of LN-10/11 chains in keratinocytes was investigated. I have developed an *in vivo* model of skin regeneration amenable to testing the long-term tissue regenerative potential and plasticity of rare keratinocyte populations (e.g. stem cells) while allowing to investigate the role of microenvironmental factors in this process. The model can easily be adapted to other epithelial tissues or used to investigate the process of tumour progression. This work was published recently in *Exp. Dermatol.*. Together, the results generated through my research have important implications for the treatment of various cutaneous pathologies including skin cancer, blistering disorders, severe burns and other chronic skin wounds.

Technical skills acquired: ³⁵S-Met/Cys-metabolic labelling, FACS analysis, immunohistochemistry, *in vitro* and *in vivo* skin regeneration assays.

Post-Doctoral Research (July 1998 – June 2000):

“Purification and characterisation of a colonic autocrine spreading factor”
Ludwig Institute for Cancer Research, Melbourne Branch

Project: This research project followed from my Ph.D. project. The aim was to purify a sufficient amount of an Autocrine Spreading Factor (ASF) from the conditioned medium of a human colon carcinoma cell line (LIM1215) to enable its identification by amino acid sequence analysis and biological/biochemical characterisation. I was able to optimise the purification protocol significantly and identified ASF by mass spectrometry, amino acid analysis and immunoblotting as laminin-10 (LN-10, $\alpha_5\beta_1\gamma_1$ trimer). Further, I demonstrated that LN-10 is a potent adhesion and motility factor for colon cancer cells and that integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ act as receptors for this laminin isoform. Taken together, the results from my post-doctoral research supports the role of autocrine TGF- α and laminin-10 in the metastasis of colon tumours.

Technical skills acquired: *In vitro* cell motility and invasion assays, proteolytic digestion, enzymatic deglycosylation, confocal microscopy.

Ph.D. Research: "Autocrine Factors Regulate Colon Cancer Cells"
Ludwig Institute for Cancer Research/University of Melbourne
Supervisor: Prof. Antony W. Burgess

Project: The aim of the research project was to study the role of autocrine factors in growth regulation using the colon carcinoma cell line LIM1215 as an *in vitro* model. I found that multiple autocrine factors including a mitogen (TGF- α) a survival/anti-apoptotic factor and an extracellular matrix (ECM)-like spreading factor are produced by LIM1215 cells and synergize to regulate their proliferation and morphology *in vitro*. I successfully purified to homogeneity a large ECM protein and work to identify this protein by amino acid sequencing was undertaken as part of my post-doctoral research project. I studied in detail the signalling pathways triggered by TGF- α /EGF stimulation of LIM1215 cells, particularly the mechanism of activation of the ras/raf-1 signalling pathway and its role in mitogenesis.

Technical skills acquired: *In vitro* cell spreading assays, immunofluorescence, ELISA, production and purification of polyclonal antibodies, sub-cellular fractionation, Western blotting, immunoprecipitation, *in vitro* kinase assays, protein purification/HPLC, electrophoresis.

M.Sc. Research: "Characterization of the Cytotoxic Activity of Guinea Pig Foa-Kurloff Cells."
University of Sherbrooke
Supervisor: Prof. Marek Rola-Pleszczynski

Project: Natural Killer (NK) cells play an important role in immunological defense and constitute the first line of resistance against viral and bacterial infection as well as tumor development. Whilst the Guinea pig has been widely used as an animal model to study natural defense against such invasion, little was known about the exact nature of the NK cell in this animal. During my M.Sc., I demonstrated that a subset of cells, previously known as Foa-Kurloff cells, were in fact the Guinea pig counterpart of human NK cells. Furthermore, I studied in detail their mechanism of action against tumor cells and showed that they display two distinct cytotoxic activities previously thought to be mediated by two subsets of cells namely, Natural Killer (NK) and Natural Cytotoxic (NC) cells.

Technical skills acquired: General cell culture, *in vitro* mitogenic and cytotoxic assays, production of monoclonal antibodies, FACS analysis.

Other Roles Undertaken:

Referee for Journals: - Growth Factors

Grant Assessor: -The Royal Women's Hospital Research Advisory Committee
-Raine Medical Research Foundation
-The Leo & Jenny Leukaemia and Cancer Foundation

Supervisor: Supervision of research assistants and students

Industry Experience:

March 1997 – February 1998 Pharmaceutical Development Project Planner
Glaxo-Wellcome Australia Ltd
1061 Mountain Hwy, Boronia
VIC, Australia 3155

July 1991- August 1992 Medical Representative
Procter & Gamble Pharmaceuticals Canada inc.
P.O. Box 355 succ A Toronto, Ontario
Canada M5W 1C5

Teaching Experience:

September 1987 – September 1989 Substitute Teacher
École Polyvalente Évariste Leblanc
1750 Mtée Masson, Duvernay Est, Laval
Québec, Canada

I taught mathematics, physics, chemistry and biology to year 7 to 12 students. In addition, I taught regular classes to students with learning difficulties. Responsibilities included the preparation and presentation of lectures, preparation and correction of exams and reports, and supervision of student's academic progress.

Publications:

1. **Pouliot N.**, K. Maghni, P. Sirois and M. Rola-Pleszczynski (1996). Guinea pig Kurloff (NK-like) cells mediate TNF-dependent cytotoxic activity: analogy with NC effector cells. *Inflammation*, 20:263-280.
2. **Pouliot N.**, K. Maghni, F. Blanchette, L. Cironi, P. Sirois, J. Stankova and M. Rola-Pleszczynski (1996). Natural killer and lectin-dependent cytotoxic activity of Kurloff cells: target cell selectivity, conjugate formation and Ca^{++} dependency. *Inflammation*, 20:647-671.
3. Walker F., A. Kato, L. J. Gonez, M. L. Hibbs, N. **Pouliot**, A. Levitzki and A. W. Burgess (1998). Activation of the Ras/MAPK pathway by kinase defective epidermal growth factor receptors results in cell survival but not proliferation. *Molecular and Cellular Biology*, 18:7192-7204.
4. **Pouliot N.** and A. W. Burgess (2000). Multiple autocrine factors including an extracellular matrix protein are required for the proliferation and spreading of human colon carcinoma cells *in vitro*. *Growth Factors*, 18:7192-7204.
5. **Pouliot N.**, L.M. Connolly, R.L. Moritz, R.J. Simpson and A. W. Burgess (2000). Colon cancer cell adhesion and spreading on autocrine laminin-10 is mediated by multiple integrin receptors and modulated by EGF receptor stimulation. *Experimental Cell Research*, 261:360-371.
6. **Pouliot N.**, E.C. Nice and A.W. Burgess (2001). Laminin-10 mediates basal and EGF-stimulated motility of human colon carcinoma cells via $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins. *Experimental Cell Research*, 266:1-10.
7. **Pouliot N.**, N.A. Saunders and P. Kaur (2002). Laminin-10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in promoting proliferation and migration. *Experimental Dermatology*, 11:387-397.
8. Jorissen R.N., F. Walker, N. **Pouliot**, T.P.J. Garrett, C.W. Ward and A.W. Burgess (2003). Epidermal growth factor receptor: mechanisms of activation and signaling. *Experimental Cell Research*, 284:31-53.
9. Zamurs A., N. **Pouliot**, P. Gibson, G. Hocking and E. Nice (2003). Strategies for the purification of laminin-10 for studies on colon cancer metastasis. *Biomedical Chromatography*, 17:201-211.
10. Li A., N. **Pouliot** and P. Kaur (2004). Extensive tissue-regenerative capacity of neonatal human keratinocyte stem cells and their progeny. *Journal of Clinical Investigation*. 113:390-400.
11. **Pouliot N.**, Redvers RP, Ellis S, Saunders NA, and Kaur P. (2005). Optimization of a transplant model to assess skin reconstitution from stem cell-enriched primary human keratinocyte populations. *Experimental Dermatology*, 14:60-9.
12. Eckhardt B.L., **Pouliot N.** and Anderson R.L. (2005). Influence of the bone microenvironment on breast cancer metastasis to bone. In: G. Meadows (ed.), *Integration/Interaction of Oncologic Growth* (In press).

Other Publications:

1. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1990).** The cytotoxic activity of the Foa-Kurloff cells. *FASEB J.*, 4:A1892.
2. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski (1990).** Étude du mécanisme lytique des cellules Foa-Kurloff. *Medecine Science, supplément no2 (99)* p.36A.

Meeting Presentations:

1. **Pouliot N., E. Sloan, A.L. Natoli, B. Eckhardt, C.M. Restall, B. Parker, L. Zamurs, E. Nice and R.L. Anderson (2005).** Microenvironmental regulation of breast tumours: evidence for the cooperative role of $\alpha v \beta 3$ integrin, laminin-10 and matrix metalloproteinase-9 in breast cancer metastasis to bone. Poster presented at the 2005 Lorne Cancer Conference, Phillip Island, Victoria, Australia.
2. **Pouliot N., C.M. Restall, A.L. Natoli, O. Narayan and R.L. Anderson (2003).** The role of MMP-9 and its regulation by tumour/stromal interactions in breast cancer metastasis to bone. Poster presented at the Fifth Peter Mac Symposium on Molecular and Cell Biology of Cancer, University of Melbourne, Victoria, Australia.
3. **Pouliot N., N.A. Saunders and P. Kaur (2002).** Laminin-10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in regulating keratinocyte cell proliferation, differentiation and migration. Oral presentation at the First Boden Research Conference on Developmental Cutaneous Biology, Stradbroke Island, Queensland, Australia.
4. **Pouliot N., A. Li, J. Karlis and P. Kaur (2002).** In vitro and in vivo tissue regenerative capacity of keratinocyte stem cells and their progeny is determined by their mesenchymal microenvironment. Poster presented at the 2002 Keystone Symposium, Keystone, Colorado, USA
5. **Pouliot N., A. Li, J. Karlis and P. Kaur (2001).** The functional role of laminin-10/11 in human keratinocyte proliferation and skin maturation. Poster presented at the Fourth Peter Mac Symposium on Molecular and Cell Biology of Cancer, University of Melbourne, Victoria, Australia.
6. **Pouliot N. A. Li and P. Kaur (2001).** Expression and function of laminin-10/11 in human skin. Poster presented at the 2001 Lorne Cancer Conference, Lorne, Victoria, Australia
7. **Pouliot N., L.M. Connolly, R.L. Moritz, R.J. Simpson and A. W. Burgess (1999).** Human colon carcinoma cells adhere to and spread on an autocrine secreted laminin-10 via integrin $\alpha_3 \beta_1$ and $\alpha_6 \beta_4$ receptors. Poster presented at the 1999 Pan-Pacific Connective Tissues Societies Symposium, Queenstown, New Zealand
8. **Pouliot N. and A. W. Burgess (1999).** Characterisation of an autocrine spreading/motility factor stimulating colonic cells. Poster presented at the 1999 Lorne Cancer Conference, Lorne, Victoria, Australia

9. **Pouliot N.** and A. W. Burgess (1996). Extracellular matrix and TGF- α are required autocrine factors required for the proliferation of the colon carcinoma cell line LIM1215. Poster presented at the 1996 Keystone Symposium, Keystone, Colorado, USA.
10. **Pouliot N.** and A. W. Burgess (1995). Multiple autocrine factors are involved in the proliferation of the colon carcinoma cell line LIM1215. Poster presented at the 1995 Lorne Cancer Conference, Lorne, Victoria, Australia.
11. **Pouliot N.** and A. W. Burgess (1994). Autocrine survival and spreading factors produced by the colon carcinoma cell line LIM1215. Poster presented at the 1994 Lorne Cancer Conference, Lorne, Victoria Australia.
12. **Pouliot N., K. Maghni, P. Sirois, and M. Rola-Pleszczynski** (1990). The cytotoxic activity of Foa-Kurloff cells. Poster presented at the 1990 FASEB Meeting, New Orleans, Louisiana, USA.
13. **Pouliot N., K. Maghni, P. Sirois and M. Rola-Pleszczynski** (1990). Étude du mécanisme lytique des cellules Foa-Kurloff. Poster presented at the 1990 CRCQ Meeting, Quebec, Canada.

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